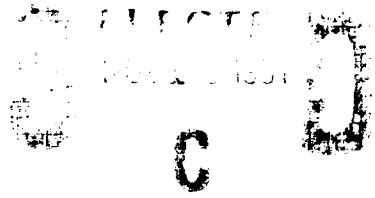

Research Progress Report

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RESEARCH PROGRESS REPORT
SUBMITTED TO THE
U.S. NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FREEZE-DRIED HUMAN RED BLOOD CELLS

CONTRACT NO. N00014-90-C-0053

CRYOPHARM CORPORATION

November 8, 1991

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SUMMARY

This progress report summarizes Cryopharm's basic red cell research since the last report submitted to the Naval Medical Research and Development Command on July 12, 1991.

As outlined in the Summary from the July 12, 1991 report, an initial clinical evaluation of in vivo circulation of autologous, lyophilized human red cells was undertaken to establish a baseline level of in vivo performance. The cell survival results suggested that the lyophilized reconstituted cells were removed intact from peripheral blood via a splenic sequestration route. We observed this mechanism of cell clearance by the body in spite of the high in vitro osmotic stability (about 80% survival of intact lyophilized cells when incubated in physiological saline) and improved cell deformability as measured by in vitro ektacytometry (lyophilized reconstituted cells exhibited some 65% of the peak elasticity normally seen in fresh red cells). Thus, although our lyophilized reconstituted red cells maintain normal levels of metabolic, cytoskeletal, and hemoglobin function, they do appear to sustain damage to their membranes which compromises deformability to an extent that their ability to pass-through small splenic or capillary sinuses is greatly reduced. Our primary project for this funding period has been to identify the nature and extent of suspected damage to the cell membrane and attempt to prevent or correct it.

In this funding period we attempted to address key issues that resulted from the initial clinical evaluation of lyophilized red cells:

1) Determine the nature of the damage incurred by the cells during lyophilization and reconstitution that targeted these cells for premature removal by the spleen. Our efforts focused on damage to the cell membrane.

2) Establish the degree of damage to the cells. If this were minor, methods to allow normal cellular repair to occur might prove sufficient to overcome the damage. If the damage was extensive, a step-by-step identification of in-process damage would be needed in order to devise methods for minimizing or eliminating the damage.

In this report we describe our experiments that addressed these key issues. As discussed in the Summary of our July 12, 1991 report, we suspected that the lyophilized cells were experiencing subtle membrane damage. This hypothesis was based on our model of transient lysis-resealing of the cells during the shock of rehydration. In this report we describe several in vitro assays that have confirmed the membrane damage hypothesis. These assays have also served to provide information on the nature of the damage and its apparent extent. Using this information, we then studied several cell treatment methods designed to assess whether the membrane damage detected in our assays could be measurably repaired. Although we conclude that the detected damage cannot be overcome by a simple repair or reannealing process, these studies did provide an estimate of the extent of damage and suggested that early stage in-process damage was

a possible contributor (i.e., we have focused on the stability of the cells when they are frozen and held within a temperature range acceptable for sublimation).

Our current studies have addressed the issue of how to eliminate cell membrane damage from our process. In order to study this issue in a logical fashion, our approach has been to dissect each process step, and evaluate the quality of the cells in a step-by-step fashion. Again, the in vitro assays, such as cell filtration, developed to study membrane damage effects, have helped monitor cell quality at each process step.

Our step-by-step process evaluation has identified the initial freezing stage as a step in which measurable cell membrane properties deviate from those of fresh red cells. This observation lead us to a theoretical basis for studying cellular stability in the frozen state. We have exploited glass transition temperature theory to predict the maximum frozen temperature at which a perishable biological product can be maintained while preventing appreciable rates of chemical reactions. This theory has already found general application in the food and pharmaceuticals (i.e., lyophilized protein) industries, and has enabled us to apply the predictive power of glass transition calculations, along with empirical studies of our buffer formulas using differential scanning calorimetry.

Our goal and future plans focus on the application of glass transition theory in buffer design to practical lyophilization. This means that our cryoprotective buffer must maintain the red cells in a stable state at the elevated frozen temperatures (above -45^{o}C) needed for efficient removal of bulk water by sublimation. We believe that this approach will enable the design of improved cryoprotective solutions that will minimize the observed instability of cells in the frozen state (a necessary condition during freeze-drying). Our next step, once the frozen stability of the cells is improved, will be to assess the consequences in in vivo studies.

UPDATE OF PROJECT STATUS RELATIVE TO 1989 MILESTONES

Cryopharm submitted its original research proposal on lyophilized red blood cells in September, 1989. In that proposal we included a chart of research milestones, and a copy of that chart is included in this section for reference.

The first listed milestone in our 1989 chart involved definition of shelf lyophilization parameters during Year 1 (May 1990-May 1991). In 1989 we did not have a defined lyophilization cycle that could yield reproducible cell quality results in different freeze-dryers. In Year 1 we successfully addressed this issue. We currently operate six shelf lyophilizer units, and each can be operated with a standard cycle that yields reproducible results (i.e., we can control sample collapse by running a specified temperature and pressure profile). Attainment of this milestone was an important step as reproducible equipment operation is key to controlled research.

CRYOPHARM CORPORATION
RESEARCH MILESTONES CHART
FREEZE-DRIED RED CELLS

Project Activities	Current Status	Milestone	Projected Start	Projected Completion
Define Shelf Lyophilization Parameters:	No defined	Defined cycle	Year 1	Year 1
Define optimal temperature, pressure conditions.	cycle	worked-out		
Evaluate sample configuration.				
Evaluate Existing Reconstitution Protocol:	~70% initial	>80% initial	Year 1	Year 1
Mixing and temperature conditions.	yield	yield		
Optimize Product Properties:				
Cell yield (at infusion stage).	~35-40%	>50%	Year 1	Year 2
Residual moisture (in dry state).	~3%	~1%	Year 1	Year 2
Final product sterility (at infusion stage).	Not done	Demonstrated	Year 1	Year 2
Shelf Life:				
Refrigerated storage.	>10 months	>2 years	Year 1	Year 3
Room temperature storage.	~2 weeks	1-2 months	Year 1	Year 3
Evaluation of Enzyme Converted Red Cells.	Not done	Initial tests	Year 2	Year 2
In vivo Animal Circulation Studies:				
Pilot studies in domestic pigs.	Not done	Done	Year 1	Year 1
GLP quality studies in domestic pigs.	Not done	If pilot tests successful.	Year 2	Year 2
In vitro Animal Red Cell Studies:	Preliminary	More samples	Year 2	Year 2
(Survey models if pig cells do not circulate)	data in.	for FDA.		
Plastic Container Development.	First prototype	Developed.	Year 1	Year 1
Streamline Reconstitution and Washes.	Not done	Underway	Year 3	To be deter.
Phase I Clinical Trials of Lyophilized Cells.	Not done	File IND	Year 3	Continues...

4(a)

1989 MILESTONES CHART

Our second milestone involved an evaluation of the 1989 rehydration protocol, in terms of mixing conditions. During Year 1 we demonstrated proof-of-concept that initial recoveries of intact red cells upon rehydration can exceed 80% of the dried cells. Although this represents an important concept validation, as described in this report we continue to refine our freezing and frozen cell stability conditions to minimize early in-process damage. We believe that reconstitution damage to the cells will need to be re-addressed once we have resolved the frozen stability of red cells in our lyophilization buffer. We plan to re-visit this issue in Year 2 (May 1991-May 1992).

Our third 1989 milestone involved optimization of certain product properties. We have explored lyophilization procedures that yield up to 50% final recovery of intact human red cells. However, we now use more sophisticated analytical methods (ektacytometry, cell transit time analyzer) not available in 1989, which suggest that the recovered cells exhibit membrane damage. Further increases in cell yield depend on resolving the membrane damage. In this report we detail our progress in membrane studies. The other milestones have passed a proof-of-concept validation. We know that our final product can attain residual moisture levels in the range of 1-2% (Karl Fisher titration assay), and product sterility for buffers, dry cells, and rehydrated ready-to-use cells has been demonstrated in 6 clinical volunteer studies. Our shelf life studies suggest that extended room temperature and refrigerated storage of the dry cells are feasible, given proper packaging.

We proposed to evaluate enzyme-converted type "O" cells during Year 2. At this time we feel it is premature to initiate this study, as further improvements are needed to reduce our in-process damage to cell membranes. However, we project that by the end of Year 2 (May 1992) our process will be sufficiently advanced to merit an initial feasibility evaluation.

In 1989 we had initial data suggesting that pig red cells may be used as an in vivo animal model. As detailed in our September 30, 1990 report, subsequent studies demonstrated that our lyophilization procedures, developed with human red cells, did not transfer to animal red cells. We explored rodent, dog, pig, sheep, monkey, and non-human primate red cells, using a variety of in vitro assays. We also tried to assess human cells in an in vivo immune-tolerant rat model, with some success. In the pig, we found a severe depletion of cytoskeletal proteins following lyophilization and rehydration, which rendered the cells extremely fragile. As a consequence, we have instead conducted low dose, autologous studies in normal human volunteers (see last milestone).

Our seventh listed 1989 milestone involved development of a sterile plastic container for lyophilization, expressly in preparation for anticipated clinical studies. As detailed in our prior reports, a successful working prototype has been built, tested, and used successfully in six clinical volunteer studies (in each case we cultured samples of the rehydrated autologous red cells for sterility; all tests showed no detectable growth in media specified for sterility testing in the Code of Federal Regulations (CFR).

We proposed to streamline the 1989 rehydration and wash procedure, which at that time involved over 2 hours of manual processing time. We have successfully adapted an automated cell washing protocol that reduces the time to about 45 minutes, and consumes only 1.5 liters of solution per unit of dry red cells. We plan to continue to refine this method, with an ultimate goal of a "no-wash" method.

Our final 1989 milestone proposed the filing of an IND for Phase I clinical trials at the end of Year 3 (May 1993). We believe that our project remains on target. Due to the absence of a useful animal model, we have already initiated low dose autologous human research in collaboration with the Department of Biomedical Research at Tufts University School of Medicine. These studies have been conducted with the appropriate Institutional Review Board approvals. The design and dosage of these studies parallels what would be used in any Phase I clinical trial. We have demonstrated the safety of the lyophilized product in six volunteers, as reported in our November 9, 1990 and July 12, 1991 progress reports.

RESEARCH PROGRESS REPORT

Background

In the Future Plans section of our July 12, 1991 progress report, we identified possible membrane damage to lyophilized reconstituted red cells as the upcoming focus of our research effort. This research focus was suggested by our in vivo observation that lyophilized autologous human red cells were removed in the spleen, combined with our in vitro measurements of reduced cell deformability and cell aggregation. These in vivo and in vitro data indicated that in-process damage to the cell membranes might cause splenic sequestration and altered membrane properties such as reduced deformability. Our strategy during this funding period has been to utilize several techniques of measuring the composition and integrity of phospholipid membranes in control versus lyophilized red cells, to directly answer this question.

The goal of our phospholipid research has been to identify the nature of the suspected membrane defects, and to qualitatively compare their extent to fresh control cells. By using this approach, we wanted to assess whether the extent of membrane damage could be easily repaired by known reannealing methods, or whether the in-process damage had to be identified, isolated, and minimized by alternative manufacturing.

Studies on Phospholipid Content and Composition in Lyophilized Versus Control Cells

In Figure 1 we measured the total phospholipid content of control non-lyophilized human red cells (CT) versus lyophilized reconstituted cells (LY). As a positive control, non-lyophilized red cells were exposed to a highly oxidizing reagent, t-butyl hydroperoxide (TB), to deliberately degrade total membrane phospholipid into by-

products that are not measured by the phospholipid assay (see Reference 1 for assay details). As shown in Figure 1, normal red cells have a total phospholipid content of about 12 ug per ml cell suspension, whereas cells oxidized by t-butyl hydroperoxide treatment are significantly depleted in total detectable phospholipid, to about 6 ug/ml cells. Lyophilized reconstituted red cells appear to be 15% depleted in total phospholipid, relative to the non-lyophilized control cells.

This experiment suggests that during the lyophilization and/or subsequent process steps, the red cells are being depleted of total phospholipid. Loss of membrane phospholipid can be explained by oxidative damage to the membranes during processing, and by loss of cell membrane by vesicle formation (loss of membrane surface area via vesicle release would correlate with the reduced Mean Corpuscular Volume, MCV, measured in lyophilized reconstituted cells; see Table 1 in the July 12, 1991 report).

We interpret these results to suggest that lysis-resealing of lyophilized red cells is occurring during the osmotic stress of rehydration. During this transient lysis-resealing, some membrane is lost by release of vesicles, thereby depleting the total phospholipid content. We believe that this issue will need to be addressed once we are prepared to study improved rehydration techniques. However, as will be seen in the following discussion, other observations on the composition of membrane phospholipids and their repair suggest that in-process damage prior to rehydration must be addressed first.

Measurements of Red Cell Phospholipid Composition

In Figure 2 we show the data obtained from a thin layer chromatographic (TLC) study of phospholipid species present in non-lyophilized versus lyophilized reconstituted red blood cells. In this experiment total membrane phospholipids extracted from the cells are separated according to their differential mobilities in the thin layer matrix (which reflects their solubility in the solvent). Known phospholipids purchased from Sigma Chemical were chromatographed in parallel on each TLC plate as standards (PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; LPE = lyso-phosphatidyl ethanolamine; PC = phosphatidyl choline; and SM = sphingomyelin). The TLC method is described in reference 2.

The data in Figure 2 suggest that no individual species of membrane phospholipid is significantly depleted in lyophilized reconstituted cells versus non-lyophilized cells. This suggests that the depletion of total phospholipid observed in Figure 1 primarily reflects a uniform membrane loss, as opposed to loss of specific phospholipids. Although these data support the concept of vesicle loss as a prime contributor to total phospholipid depletion in lyophilized cells, other alterations to the membrane lipids cannot be ruled out. Our studies with fluorescent membrane probes address this issue.

Merocyanine 540 Dye Labeling of Intact Red Cells

The fluorescent dye merocyanine 540 has been used as a probe for the integrity of phospholipid membranes (reference 3). The dye molecule comprises a planar, aromatic

ring structure that can insert between the hydrophobic phospholipid "tails" in a membrane. The amount of dye binding depends upon how tightly packed the phospholipids are in the membrane. Normal red cells exhibit a characteristic level of dye binding, indicative of normal membrane integrity, whereas damaged or leaky membranes exhibit increased dye binding. As an example, cells deliberately damaged by treatment with lipid oxidizing agents such as t-butyl hydroperoxide exhibit increased dye binding and fluorescence. In this experiment we used a modified technique from Allan et al., involving butanol extraction of membrane-bound dye to ensure complete recovery of bound dye from the washed cell pellet.

In Figure 3 we measure the amount of merocyanine 540 binding to red cells subjected to various treatments. Total fluorescence intensity (expressed in arbitrary units) measured in a spectrofluorimeter is seen to increase with treatments known to damage cell membranes. Control (CT) non-lyophilized cells exhibit a baseline level of dye binding. Treatment of normal red cells with the oxidants phenazine methosulfate (PH) or t-butyl hydroperoxide (TB) cause increased dye binding. Phenazine methosulfate is known to attack both the protein and lipid components of a cell membrane, while t-butyl hydroperoxide is more effective against the phospholipid component. Lyophilized reconstituted red cells (LY) also exhibit elevated dye binding, indicative of a disruption of the lipid packing in the membranes.

From these data we conclude that lyophilized reconstituted red cells are not only depleted in total phospholipid content, presumably due to lysis-resealing and vesicle loss during rehydration, but also may contain distortions in the packing of their membrane phospholipids. These distortions in membrane structure may in part explain the observed loss of membrane deformability, and may contribute to splenic clearance in vivo.

As part of this experiment, we attempted to gauge whether simple incubation of lyophilized rehydrated red cells in resealing buffers might alleviate some of the observed membrane perturbation. In Figure 3, Treatment A consisted of incubating lyophilized reconstituted cells in PIGPA buffer (phosphate, inosine, glucose, pyruvate, adenine), which has been used to rejuvenate deglycerolized frozen red cells. In Treatment B we devised a PIGPA-based resealing buffer with an added cholesterol-based compound which we thought may improve the resealing and phospholipid packing of the cell membranes. Neither treatment appeared to significantly improve the cell membrane integrity, as monitored by the merocyanine assay.

Single Cell Measurements of Merocyanine Fluorescence Intensity by FACS

In order to validate the observations in Figure 3 on an individual cell basis, we coupled the merocyanine 540 dye assay with fluorescent-activated cell sorting (FACS), using a flow cytometer. Using this instrument, it is possible to analyze different cell populations treated with merocyanine 540 to obtain a fluorescence intensity profile derived from measurements on thousands of individual cells. The FACS technique is described in Reference 4.

In Figure 4 the number of cells (x 100) is plotted on the ordinate versus fluorescence intensity, such that each plot shows the cell number distribution across a range of fluorescence (i.e., dye binding). In panel 4A is the fluorescence intensity distribution of a non-lyophilized sample of red cells (peak fluorescence intensity = 374). Panel 4B shows a similar distribution for a sample of lyophilized reconstituted cells (peak intensity = 468). These data support the prior observations in Figure 3, based on individual cell measurements.

In panel 4C we pretreated a sample of red cells and lyophilization buffer with carbon monoxide and nitrogen gas, respectively, to inhibit oxidation damage. Purging the buffer with nitrogen gas reduces dissolved oxygen in the solution, while exposing red cells to carbon monoxide purges bound oxygen from the cellular hemoglobin. Since carbon monoxy-hemoglobin cannot carry oxygen, this experiment was only designed to assess the effects of trying to thoroughly purge oxygen from the system before lyophilization. As shown in panel 4C, we did observe that cells processed and rehydrated in an oxygen-poor environment did exhibit a peak fluorescence intensity closer to that of non-lyophilized cells (peak intensity = 357). This study suggested that some benefit may be derived by at least minimizing the amount of dissolved oxygen in the processing buffers. Since carbon monoxide binds hemoglobin more tightly than oxygen, we do not see a useful in-process application for CO gas purging.

Assessment of Cell Membrane Permeability Using Fluorescein Diacetate

The data in Figures 3 and 4, which exploit the properties of merocyanine as a probe of membrane integrity can be extended using the charged dye fluorescein diacetate. This negatively charged dye molecule normally does not permeate the negatively charged phospholipid bilayer of an intact cell membrane. However, damaged and leaky cell membranes will allow uptake of the dye into the cell (reference 5). We have used uptake of fluorescein diacetate into red cells as a relative measure of their membrane "leakiness".

In Figure 5 we show the fluorescence intensity measured in non-lyophilized (CTR) control cells versus lyophilized reconstituted cells (LYO). These data clearly show increased permeability of the dye in the lyophilized cells, which we interpret as due to membrane leakiness. This result was expected given the data in Figures 3 and 4.

In Figure 5, Treatment A, we examined the ability of polyphosphate to inhibit cell leakiness and lysis. Certain membrane permeable polyphosphates, such as inositol hexaphosphate (IHP), are known to stabilize red cells against membrane loss and hemoglobin leakage by binding to the cell cytoskeletal proteins and presumably stabilizing the membrane. Red cells exposed to lyophilization buffer containing polyphosphate (Treatment A) clearly retain an improved ability to exclude fluorescein diacetate following reconstitution. Unfortunately, our earlier work with polyphosphates indicated that compounds such as IHP compete avidly for the 2,3-DPG binding site on hemoglobin, creating a right-shift effect in the P-50 oxygen dissociation curve and more

importantly, leading to an apparent destabilization of the molecule (IHP treatment leads to significantly increased levels of methemoglobin and hemichrome).

We believe that once improved lyophilization conditions are developed, the use of polyphosphates or other compounds can be re-examined for their ability to aid red cells to withstand the stress of rehydration. Before such work can be effective, however, in-process damage during the earlier freezing stages must be controlled. As will be seen in a subsequent section, our work on red cell membrane damage has identified defects that appear during frozen storage of the cells. We believe that the first issue to be addressed is to stabilize the cells against damage in the frozen state, as this is a necessary initial state during freeze-drying.

Evaluation of Membrane Resealing Treatments Using a Cell Filtration Assay

Our studies using fluorescent dyes to probe the structure and integrity of the cell membrane in lyophilized reconstituted cells indicated that the phospholipid bilayer of these cells is detectably altered from that of fresh red cells. We believe that one consequence of this alteration is reflected in the increased permeability or leakiness of the cells to charged dyes such as fluorescein diacetate. The next question we wished to address was whether the membrane properties of lyophilized cells could be treated after reconstitution to repair the observed damage.

Our approach to this problem is based on our model of transient lysis-resealing of the cell membranes, which presumably occurs at least during the stress of rehydration. The observed leakiness detected by fluorescein diacetate could result from partial resealing of the cell membranes. In these experiments we explored the use of various compounds reported to increase the efficiency of membrane resealing after hypotonic lysis of red cells (Reference 6). This approach is based on the assumption that repair of hypotonic lysis of red cells may provide insights on treatments that could be used to repair lyophilized reconstituted cells.

In order to measure the effects of various resealing treatments, we adopted a cell filtration assay to mimic the physical deformation that red cells undergo during their passage through the spleen and microvasculature. Whereas ektacytometry provides a measure of cell deformability in response to shear stress in a viscous solution, the cell filtration assay challenges the cells to traverse a narrow pore. We reasoned that a mechanical filtration assay would serve as a useful in vitro measure of whether repair treatments were having a meaningful effect on the cell membranes.

We used a filtration device known as a Cell Transit Time Analyzer (Reference 7). This device utilizes hydrostatic pressure to drive a suspension of red cells through a filter having 5 micron diameter pores. Each filter contains about 30 pores, and electrical conductivity between the saline solutions on either side of the filter is used to monitor the passage of individual cells through the pores (a normal discocytic red cell has dimensions on the order of 3 x 5 microns). In this assay, fresh red cells having normal deformability exhibit a mean per cell transit time of about 3 milliseconds. Cells that have suffered

membrane damage, and consequent loss of deformability (or increased membrane rigidity) will exhibit prolonged mean cell transit times. In addition, calculation of the percent covariance in the observed mean transit time for each sample can provide information on the heterogeneity of the cell population with respect to deformability.

In Figure 6 we show the mean transit time observed for different samples of human red cells. The covariance for each of these samples is plotted in Figure 7. Fresh control cells (CT) exhibit a mean transit time of about 3 milliseconds, with a low covariance. This indicates that fresh red cells behave as a fairly homogeneous cell population, with a tight distribution around the mean transit time value (i.e., the population mean closely reflects the per cell transit time, and we interpret this as a reflection of comparable deformability in most of the cells in a fresh sample).

Lyophilized reconstituted cells exhibit a longer mean transit time of about 4.5 milliseconds, and also exhibit a significantly higher degree of sample covariance. We interpret these data as suggestive of increased heterogeneity in these cells relative to fresh cells. We believe that the lyophilized reconstituted samples consist of subpopulations of cells with varying degrees of membrane damage, and hence exhibit a broader distribution of deformability and ability to traverse the filter pores.

We then used the cell filtration assay to explore the effects of several repair treatments on lyophilized reconstituted cells. In Figures 6 and 7, Treatments A-F examined several strategies to reverse the observed defects in lyophilized cells:

A) In this treatment the lyophilized red cells were directly reconstituted in a PIGPA-based (phosphate, inosine, glucose, pyruvate, adenine) rejuvenation buffer. We wished to determine whether priming the viable metabolic pathways in the reconstituted cells could enhance cellular membrane repair systems.

B) In this treatment cells reconstituted in our standard rehydration buffer were then further incubated at 37°C in the PIGPA-based rejuvenation buffer.

C) In this treatment lyophilized cells were reconstituted in PIGPA-based buffer and then further incubated at 37°C in PIGPA supplemented with an antioxidant shown in earlier studies to improve membrane deformability.

D) Lyophilized cells were reconstituted in PIGPA-based buffer and then treated with PIGPA with 5mM of membrane crosslinking reagent. This treatment was intended to assess whether mild crosslinking may provide some stability, although such treatment might be expected to also increase the rigidity of the cells.

E) Lyophilized cells were reconstituted in PIGPA-based buffer and then incubated at 37°C in PIGPA containing a membrane intercalating agent that can increase the fluidity of phospholipid bilayers.

F) Reconstituted cells were incubated in a modified hypertonic (1000 mOsmol) PIGPA solution at 37°C to minimize the initial degree of cell swelling.

As seen in Figure 6, these post-lyophilization repair approaches did not significantly reduce the mean cell transit time. In addition, as seen in Figure 7 the percent covariance in samples subjected to these treatments remained significantly above normal. We concluded from these studies that a simple, post-lyophilization repair strategy could not overcome the damage incurred to the cell membrane during the preceding process steps. Although we have not tested these conclusions in an in vivo survival study, we believe that the degree of cell heterogeneity in our samples as measured by the filtration assay argues for a different approach to the problem.

Evaluation of Cell Stability in the Frozen State

In the preceding experiments, we used several methods to address the biochemical properties of membranes in lyophilized reconstituted cells (i.e., their phospholipid content and composition), and to measure the structural integrity of the cell membranes by dye binding, dye permeability, and mechanical filtration. All of these studies suggested that the membranes of lyophilized reconstituted red cells are measurably perturbed, and that simple post-process repair strategies do not appear to offer a promising solution. Based on these conclusions, we decided that a step-by-step evaluation of our existing lyophilization process was needed to pinpoint specific steps at which harmful membrane effects could be detected. Since the lyophilization process requires that the cells be first frozen and then maintained in a stable frozen state during sublimation, we reasoned that the frozen stability of our cells in lyophilization buffer would be a logical starting point.

The stability of cells at a selected frozen storage temperature is important in the lyophilization process because efficient sublimation of water ice can only be accomplished when the sample temperature is at least -45°C or higher. As shown in the diagram in Figure 8, the vapor pressure of water vapor increases with the temperature of the ice. Removal of water vapor by freeze-drying requires a measurable vapor pressure, and this process becomes more efficient for higher vapor pressures. A practical limit for freeze-drying occurs at about -45°C, since below that temperature the vapor pressure of water is too low to permit sublimation to occur on a realistic time scale. This means that a cryoprotectant buffer for lyophilization must maintain frozen red cells in a stable condition at temperatures above -45°C.

In Figure 9 we show the results of a freezing and thawing experiment conducted with human red cells suspended in Cryopharm lyophilization buffer. We use an "E value" to measure the overall yield and quality of the recovered cells (E = mean cell number recovery post-thawing x mean osmotic stability/100). For example, in our hands red cells frozen in 40% glycerol and then thawed have measured E values of about 78. In this experiment we stored different samples of red cells frozen in lyophilization buffer at different storage temperatures for at least 15 hours, and then measured the E

value of each sample after thawing. As seen in Figure 9, our lyophilization buffer keeps the cells in a stable frozen state at temperatures below -50°C, but that significant decreases in the quality of the thawed cells occurs after frozen storage at higher temperatures. These data suggest that one possible source of the observed membrane damage in lyophilized cells could actually result from decomposition while the cells are undergoing sublimation in the frozen state.

In Table 1, more detailed data are presented on the stability of red cells stored frozen in lyophilization buffer at two temperatures, -80°C and -38°C. From the data in this Table, it can be seen that the osmotic deformability profile of frozen-thawed cells stored at -38°C is also abnormal, with a marked reduction in the peak deformability (DI max) measured by ektacytometry. We interpret these results as also pointing to membrane damage occurring while the cells are frozen.

Introduction to Glass Transition Theory

Based on our studies of frozen red cell stability, we concluded that a modified lyophilization buffer would be needed to maintain the frozen cells in a stable state at the elevated frozen temperatures required for lyophilization (i.e., above -45°C). Glass transition theory has been developed to predict the physico-chemical behavior of many natural and synthetic polymer systems, particularly for systems in which water is the solvent (see Reference 8 for a review). In Figure 10 we illustrate the application of glass transition theory to a hypothetical polymer system. The phase diagram shows the freezing and glass transition curves for this system (SOL=solution phase; ICE & SOL=mixed ice/solution; R=rubbery state; G=glass state; TE=eutectic temperature for crystalline formation; TG=glass transition temperature for amorphous glass formation). The essential principle is that when the system is maintained at temperatures below the glass transition curve, chemical reactions that require molecular diffusion are eliminated. In a fully hydrated system (low % solids), the system must be kept at very low temperatures to remain below TG; under the proper conditions an amorphous (non-crystalline) state is established in which very high viscosities prevent molecular diffusion on practical time scales. Thus, degradative chemical reactions, such as oxidation of membrane phospholipids, are eliminated. A second principle is that as the system is dehydrated (for example by sublimation), the glass transition temperature rises, so that at high % solids the system can tolerate ambient temperature storage.

The glass transition temperature for any aqueous system can be estimated based on the weight percent composition of the starting dissolved solids. By applying this concept to the design of our lyophilization buffers, we can devise formulations that should have elevated glass transition temperatures in the range of -45°C to -10°C that are useful in lyophilization. We believe that this approach will guide us in the development of buffer formulations that will circumvent the observed cell instability at frozen temperatures above -50°C.

The practical application of glass transition temperature can be illustrated with glycerol frozen red cells. Aqueous glycerol (40% w/v) has a glass transition temperature of about -65°C. This explains the current requirement to store glycerolized red cells at low temperatures. If the temperature is allowed to rise above about -65°C, then the viscosity of the amorphous glass drops exponentially, allowing molecular diffusion and chemical reactions to occur. The consequence is that glycerolized red cells stored frozen above -65° begin to exhibit all of the same problems (reduction in cell recovery, reduced osmotic stability and deformability) observed in Figure 9.

Application of Glass Transition Theory to Buffer Design

We have applied the concepts discussed above to the formulation of trial lyophilization buffers, with the initial goal of preventing measurable decreases in the quality of red cells maintained in the frozen state at temperatures useful for sublimation. We have devised several buffer formulations with estimated glass transition temperatures (based on theoretical calculations) that exceed -45°C. We have also set-up a differential scanning calorimeter to measure the glass transition temperature of each cell/buffer suspension. If prolonged high temperature frozen storage of red cells can be achieved without associated cellular degradation, this will lead to the next step of lyophilizing the frozen composition. To enable practical lyophilization cycles, the buffer system will need to preserve the frozen cells for at least 3-4 days of primary drying, during which the bulk of the water in the crystalline ice phase is removed. As the water is removed, the glass transition temperature (TG) will increase; hence the later stages of the lyophilization process will proceed more rapidly as more heat can be delivered to the system without exceeding TG.

In Figure 11 a preliminary application of this approach is shown. We devised four lyophilization buffer formulas having the following calculated glass transition temperatures (each TG was calculated for the appropriate cell/buffer dilution): Buffer 1 = -30.7°C; Buffer 2 = -32.1°C; Buffer 3 = -30.7°C; and Buffer 4 = -31.8°C. The measured glass transition temperatures were obtained for each buffer at the same dilution, using differential scanning calorimetry, and verified to be within a few degrees of the calculated values. Human red cells were suspended in each buffer to attain the desired cell:buffer ratio, then frozen to a starting temperature of -80°C, and then stored for at least 30 minutes at a specified storage temperature (-55, -45, -30, and -20°C). Each frozen sample was then thawed and the osmotic stability and cell number recovery determined to derive an "E value" (as a reference, red cells stored at -80°C in glycerol have an E-value of about 78). The results in Figure 11 show that our trial buffer systems maintain the frozen stability of the red cells at -55°C. Buffer 1 appears to give the best results, and permits frozen storage as high as -45°C, which we view as a practical threshold temperature for lyophilization. As expected, storage of the red cells at temperatures above the calculated TG yield reduced cell quality. This initial experiment provided a first validation of the application of this approach to an early process step in the lyophilization cycle.

An interesting observation in Figure 11 is that Buffers 2-4 failed to maintain the frozen storage stability of red cells at -45°C, although this temperature should lie below the glass transition temperature of each composition. We believe that this is a reflection of other effects, such as solute concentration in the frozen glass surrounding the cells, that may damage the cell membrane. Thus, we can use glass transition theory to estimate a good starting point for each buffer formulation, although other strategies will be needed in buffer development. These strategies are discussed in the Future Plans section. However, it is important to note that any contemplated formulation can first be tested to see whether it meets the high glass transition requirements for lyophilization.

FUTURE PLANS

In our studies of frozen red cell stability, based on glass transition temperature, we observed that buffered solutions having high glass transition temperatures are necessary but not sufficient to maintain the stability of the cells in the frozen state. Other researchers (References 9, 10) have indicated the existence of critical frozen transition temperatures above which red cell hemolysis occurs. Chaplin and Schmidt (1957) and Valeri and Bond (1967) (References 11, 12) demonstrated that human red cells preserved with a glycerol-slow freeze method were stable for 3 days at -20°C and up to 7 days at -30°C. Storage in glycerol for longer periods at -20°C resulted in excessive hemoglobin loss and reduced in vivo survival. Unfortunately, glycerol cannot be used in lyophilization due to its low melting point and lack of ice crystal formation. Meryman has suggested several mechanisms by which freeze-induced cellular damage could occur (Reference 13). One such mechanism invokes excessive cell dehydration during freezing as solutes and cells become increasingly concentrated into the amorphous glass phase and are excluded from the crystalline ice phase.

We suspect that such solute effects in the glassy phase may contribute in part to the observed instability of cells stored frozen at high temperatures. These effects can involve both the dissolved salts as well as the pH of the glass phase. In addition, our studies to date are based on measured glass transition temperatures of the cell suspensions (i.e., the extracellular medium). We do not know what conditions exist inside the red cells, and in fact the total intracellular volume is a significant portion of the whole solution. We plan to modify our existing high glass transition temperature buffers to address these concepts, and to study whether frozen cell damage can be reduced. Several modifications can be explored:

1) Use of increased concentrations of permeant cryoprotectants to minimize formation of intracellular crystalline ice and solute concentrates during freezing. This in turn should lessen the solute-concentrating effect of ice formation.

2) Use of different buffer salt compositions to maintain pH to within a narrow range may minimize pH effects caused by freeze-concentration of solutes.

3) Evaluation of small molecules capable of rapid membrane permeation to augment the action of carbohydrates in preservation of the membrane phospholipids.

4) Tests on the effects of membrane stabilizing drugs that may prevent phospholipid phase transitions during freezing, drying, or rehydration. These drugs may provide valuable insights on the relevant physical-chemical mechanisms.

5) Evaluation of freezing rate conditions and ultimate freezing temperature, as another approach to minimizing the formation of freeze-concentrates.

We plan to devote the next funding period to obtaining a better understanding of the important parameters that effect the stability of red cells in the frozen state. Our goal will be to develop buffer systems capable of maintaining the starting condition of the cells at elevated frozen temperatures useful for sublimation. A 3-4 day frozen storage stability at temperatures above -45°C will permit subsequent studies to then address the ease of lyophilization of such compositions. The next stage in our step-by-step process dissection will be to evaluate the condition of the rehydrated cells. If the critical cell membrane parameters, such as membrane permeability, phospholipid composition, and membrane deformability can be recovered, the performance of these cells can then be determined by in vivo survival.

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FIGURE 1. Total Phospholipid contents of non-lyophilized cells (CT), lyophilized (LY) and t-butylhydroperoxide treated cells (TB)

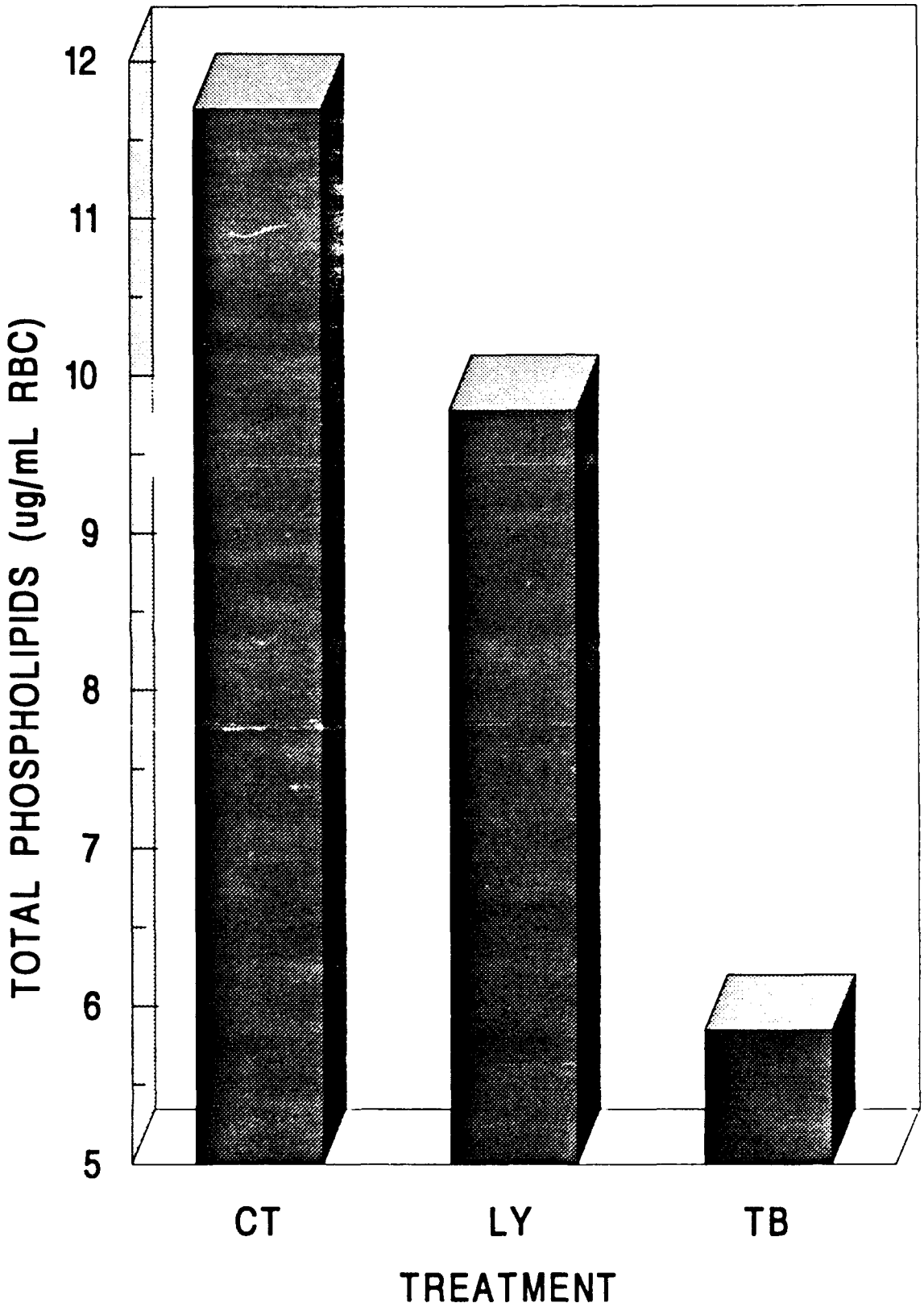

FIGURE 2. Individual phospholipid components of human red blood cells separated with TLC assay on silica-gel plates.

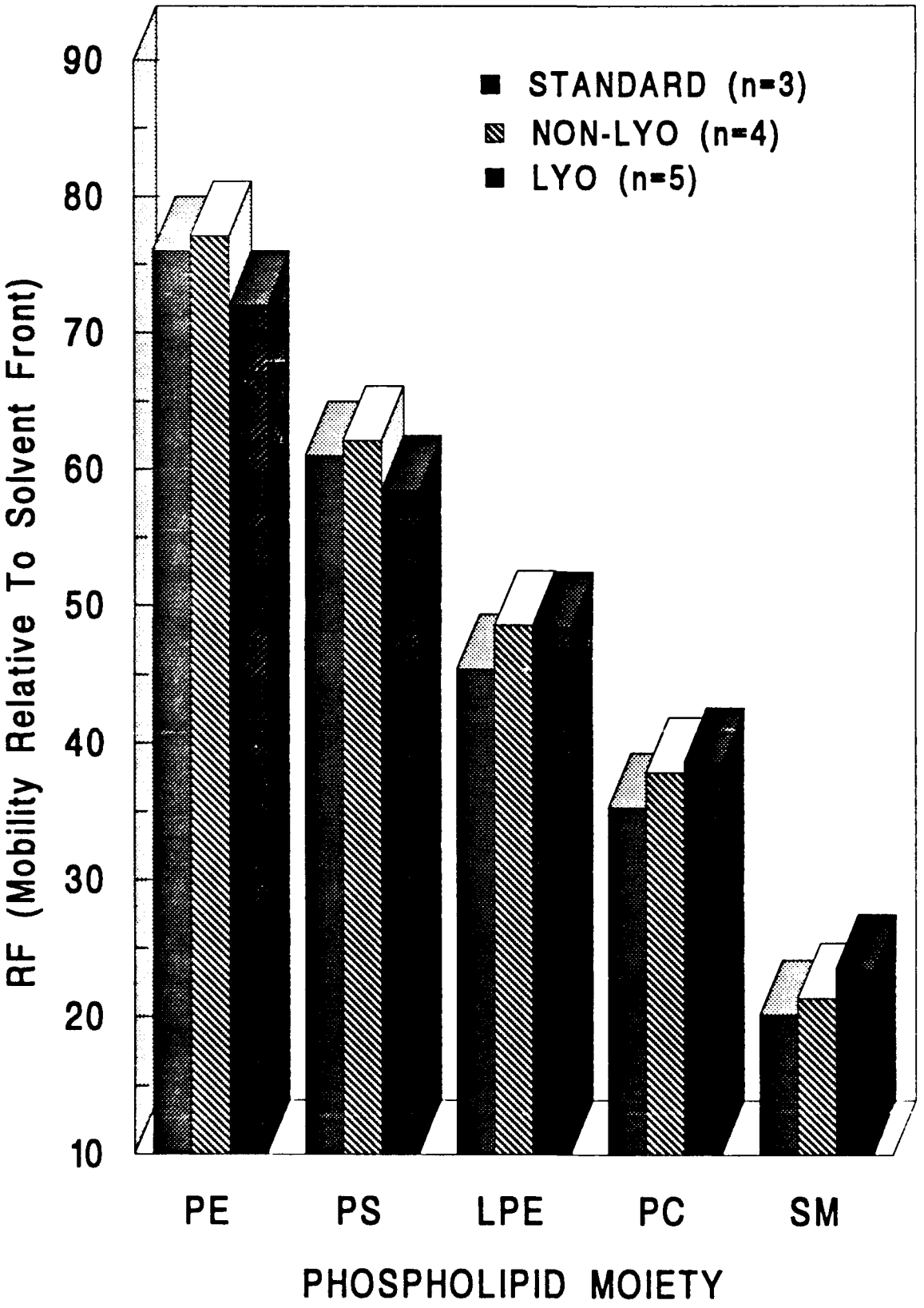

FIGURE 3. Fluorescence intensity profiles of RBC labeled with merocyanine 540 (MC540).

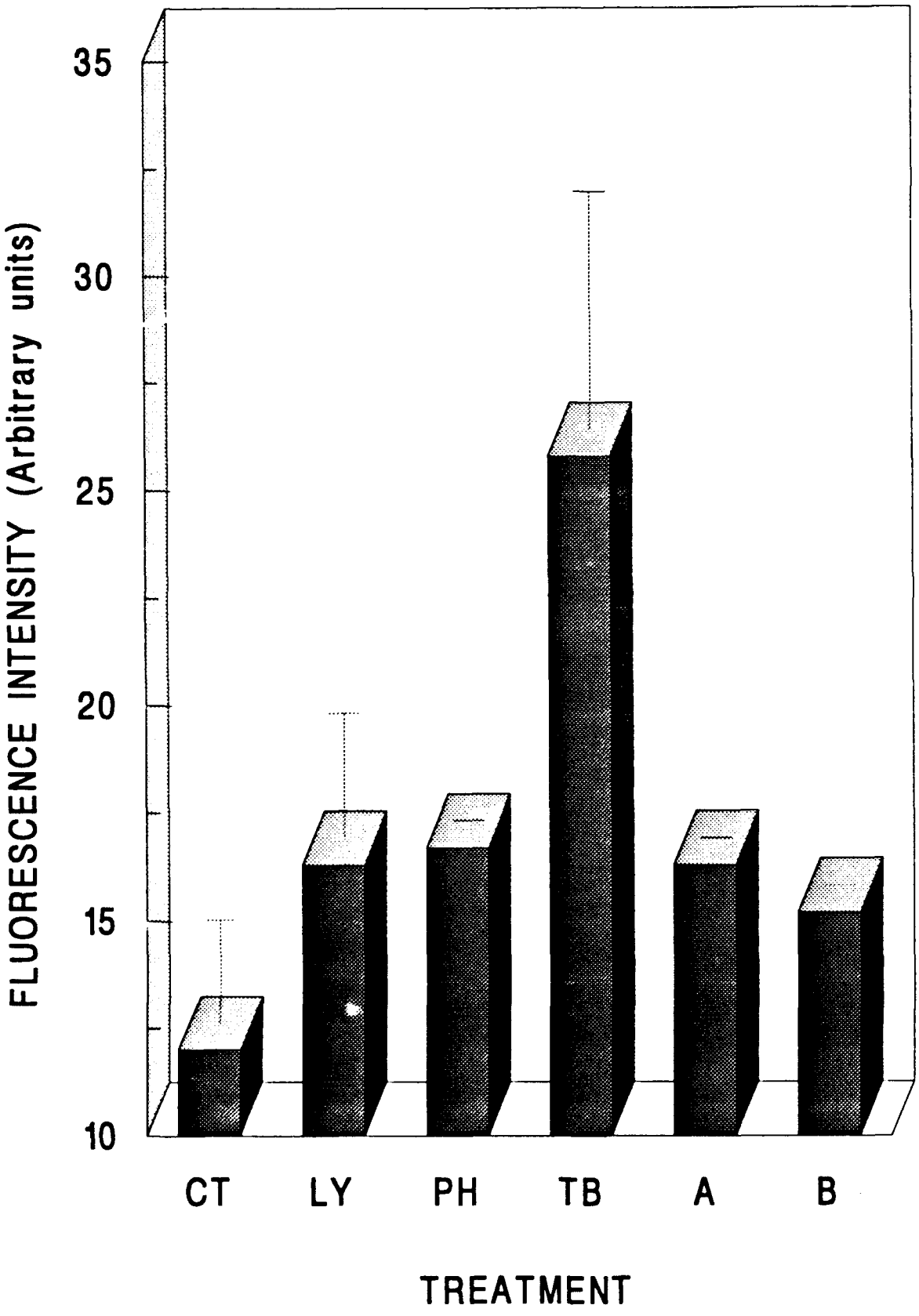

FIGURE 4

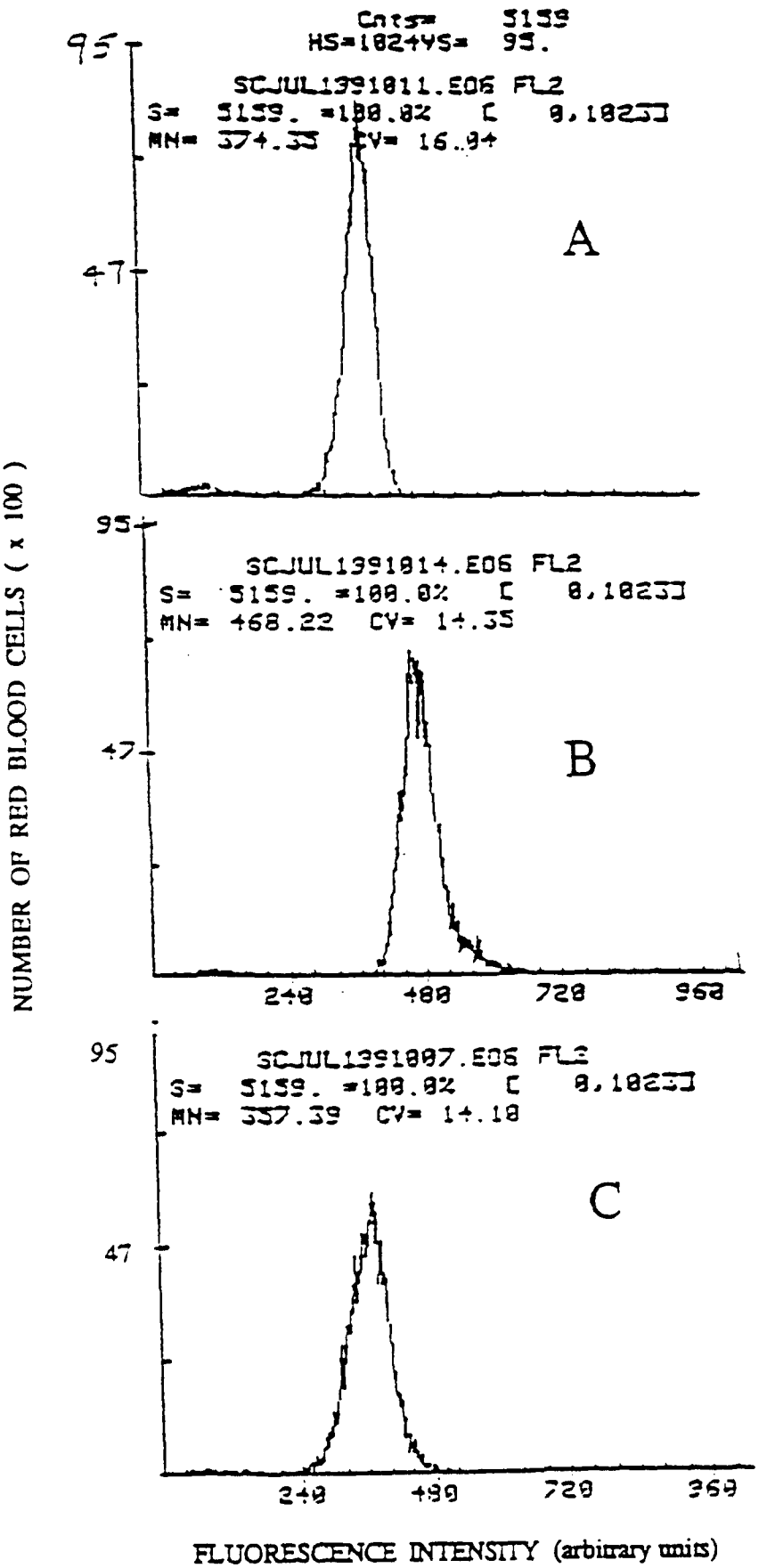
Cnts= 5159
HS=1024YS= 95.
95
SCJUL1391811.E06 FL2
S= 5159. =100.0% C 0.10233
MN= 374.33 CV= 16.94
47
A
95
SCJUL1391814.E06 FL2
S= 5159. =100.0% C 0.10233
MN= 468.22 CV= 14.35
47
B
240
480
720
960
95
SCJUL1391807.E06 FL2
S= 5159. =100.0% C 0.10233
MN= 357.39 CV= 14.18
47
C
240
480
720
960
NUMBER OF RED BLOOD CELLS (x 100)
FLUORESCENCE INTENSITY (arbitrary units)

FIGURE 5. Fluorescence intensity profiles of RBC labeled with fluorescein diacetate.

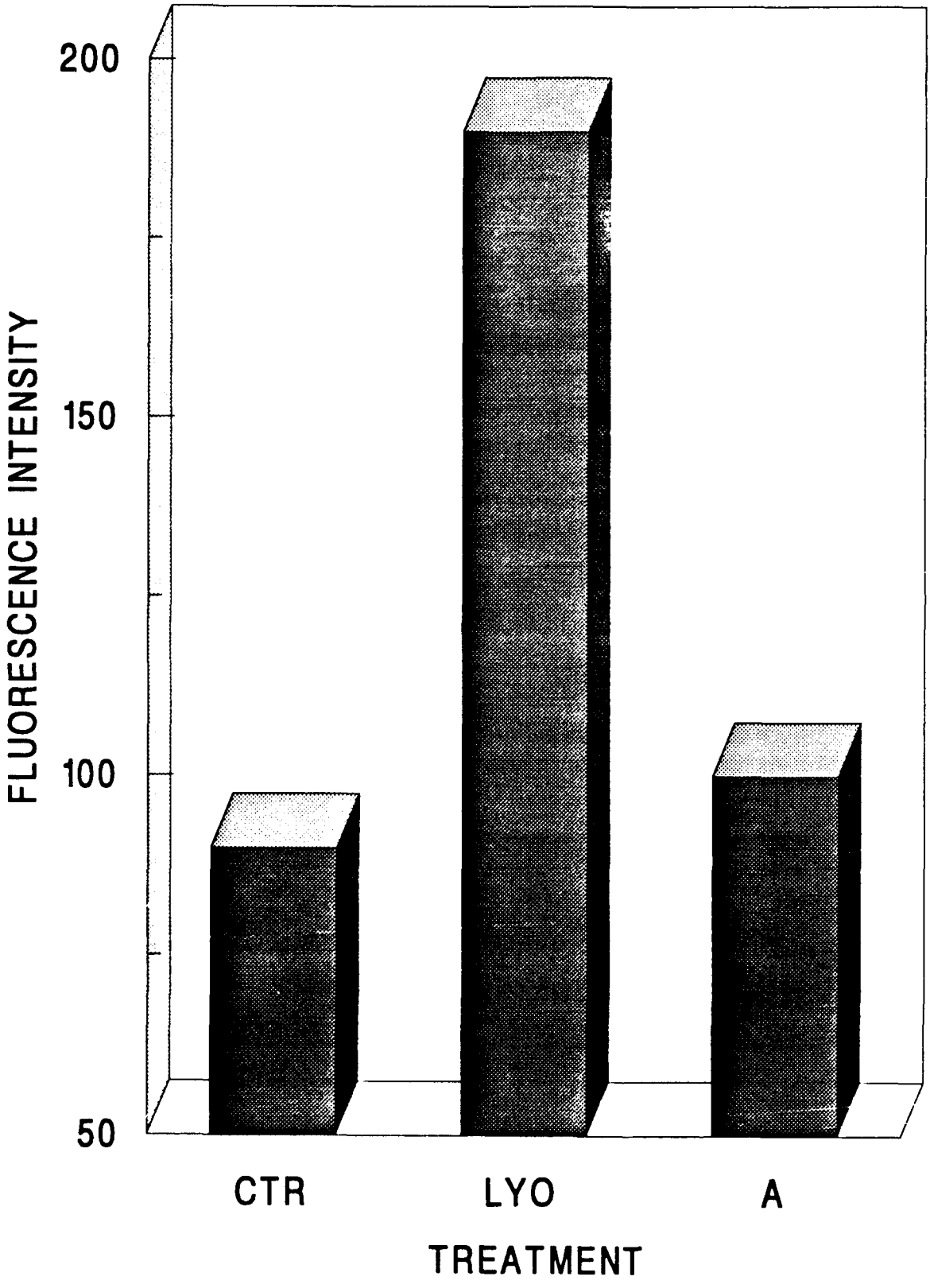

FIGURE 6. Mean transit times of control (CT), lyophilized RBC with different treatments (L-F)

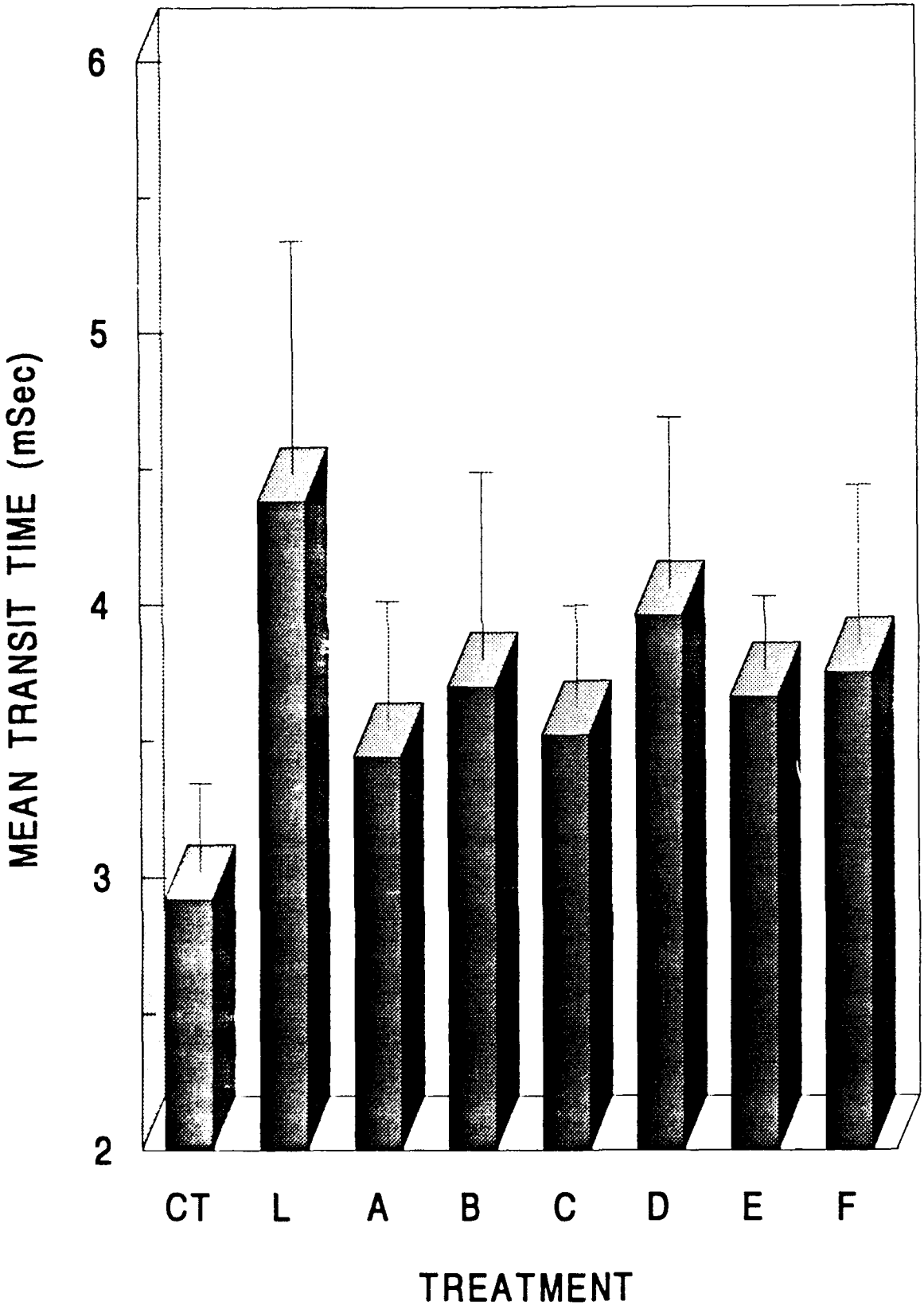

FIGURE 7. Covariance of the mean transit times of normal RBC (CT) and lyophilized RBC (L-F)

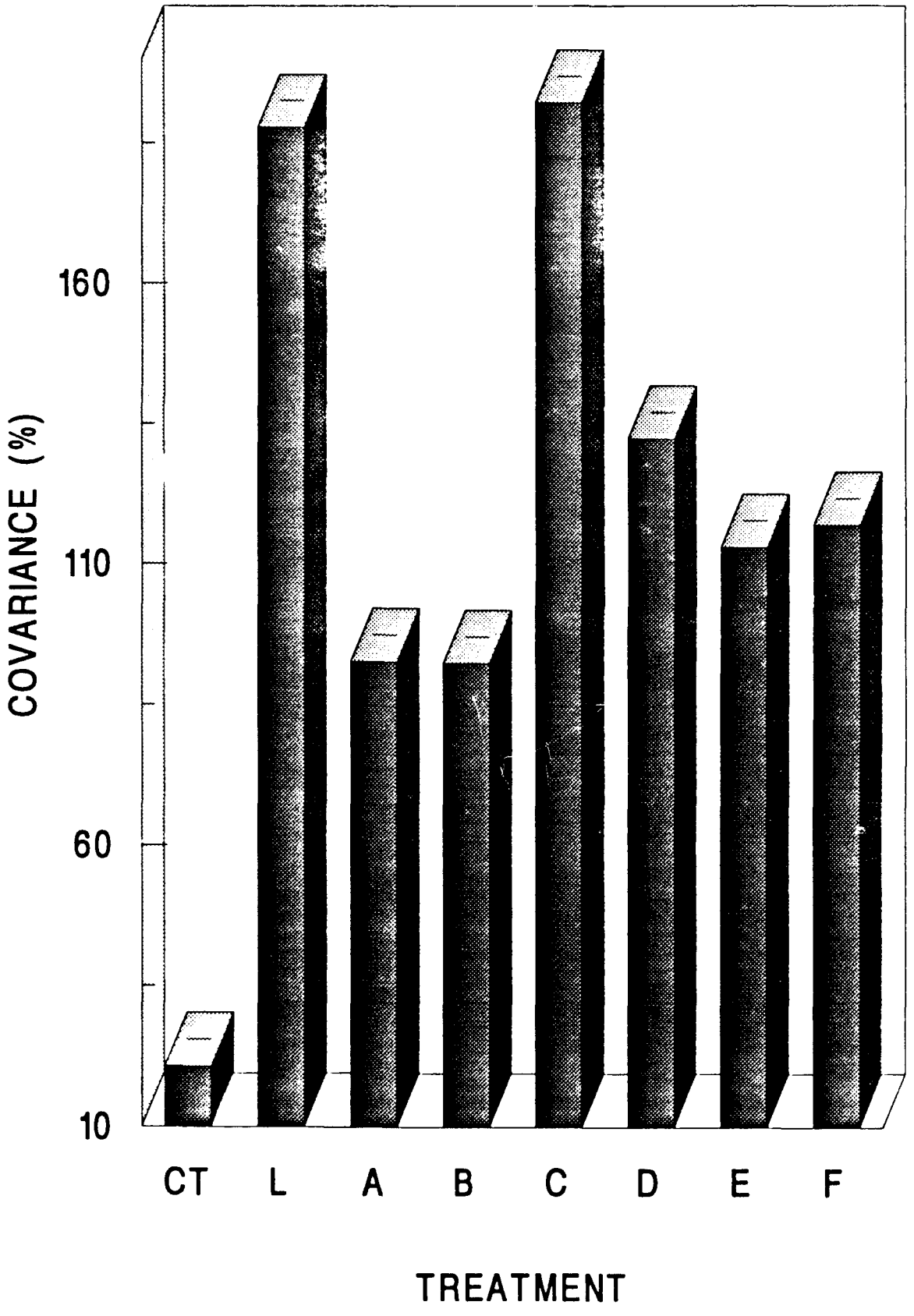

FIGURE 8

VAPOR PRESSURE AS A FUNCTION OF ICE TEMPERATURE

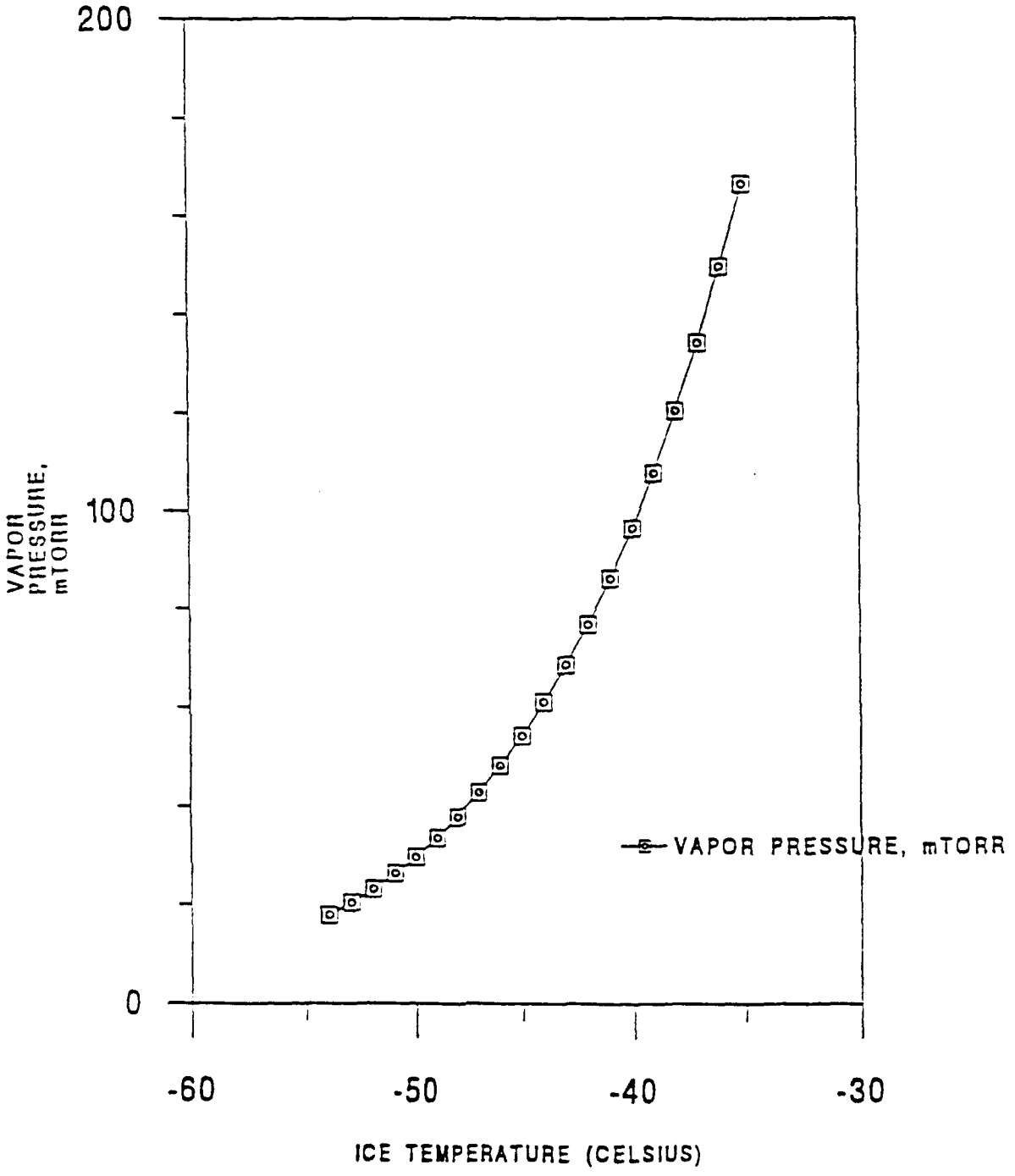

FIGURE 9

VARIATION IN SAMPLE QUALITY WITH STORAGE TEMPERATURE

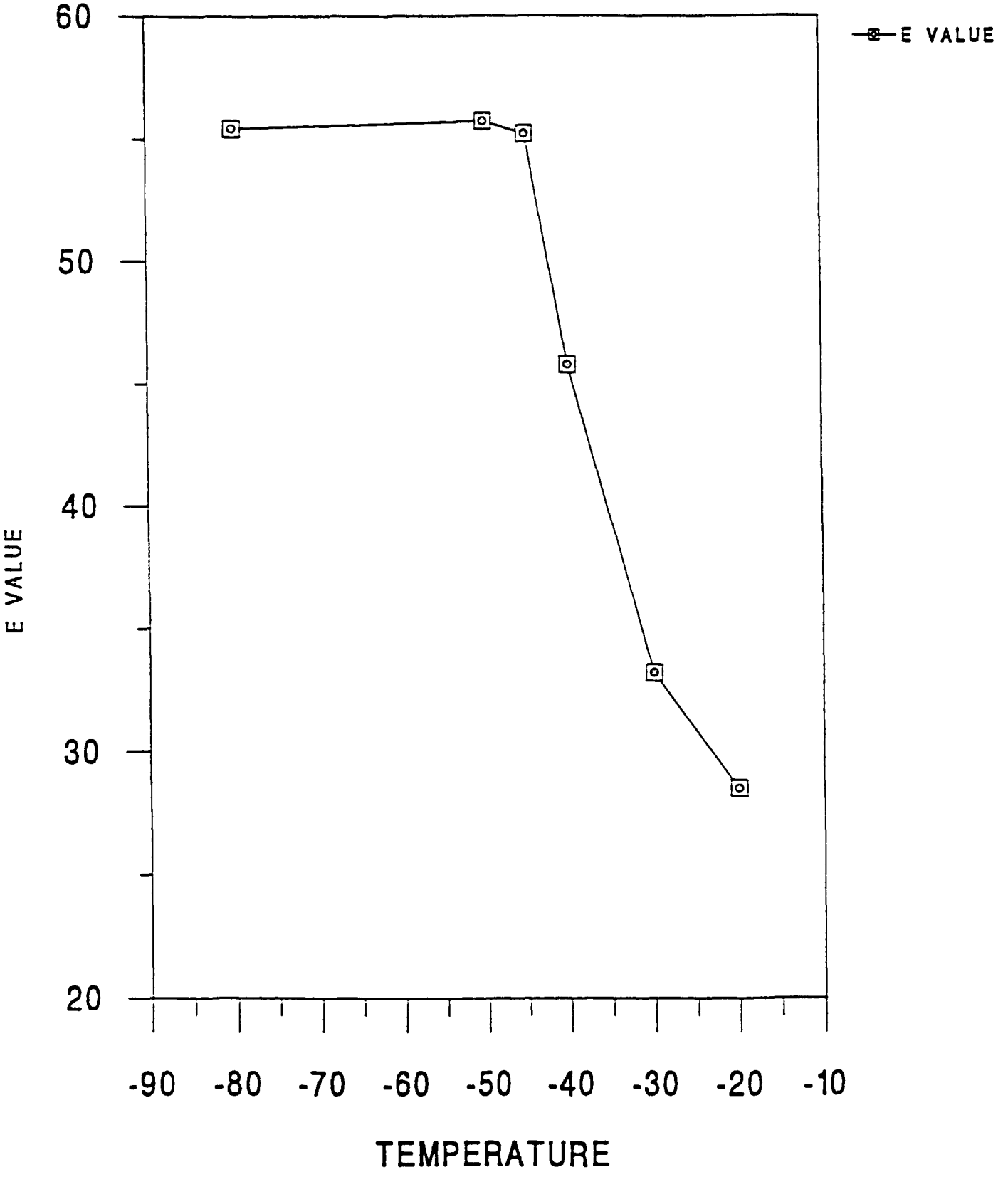

Table 1. Summary of the expected target values of all the characteristics of red blood cells. Note that the red blood cells were frozen at - 80C and then stored at either -80C or - 38C for 15 hours

CHARACTERISTICS OF NORMAL RED BLOOD CELLS	EXPECTED VALUES	BUFFER # 1 AT - 80C	BUFFER # 1 AT - 38C
Recovery at Reconstitution	90% or greater	97.6	86.9
Overall Cell Recovery	80% or greater	92.7	70.0
Osmotic Stability of Cells	70% or greater	74.6	45.8
Overall Quality of Cells (E)	56.0 or greater	69.2	32.1
Mean Cell Volume (fl)	80-100	97.0	102.8
Mean Cell Hemoglobin (pg)	25-35	31.8	31.2
Mean Cell Hemoglobin Concentration (g/dL)	31-37	32.8	30.4
Maximum Deformability Index	0.500 or greater	0.514	0.427
% of Control Maximum Cell Deformability	80% or greater	80.7	67.0
Osmotic Deformability Profiles	NORMAL	NORMAL	ABNORMAL
Density of Cell (g/mL)	1.095	1.101	1.101

FIGURE 10

PHASE DIAGRAM

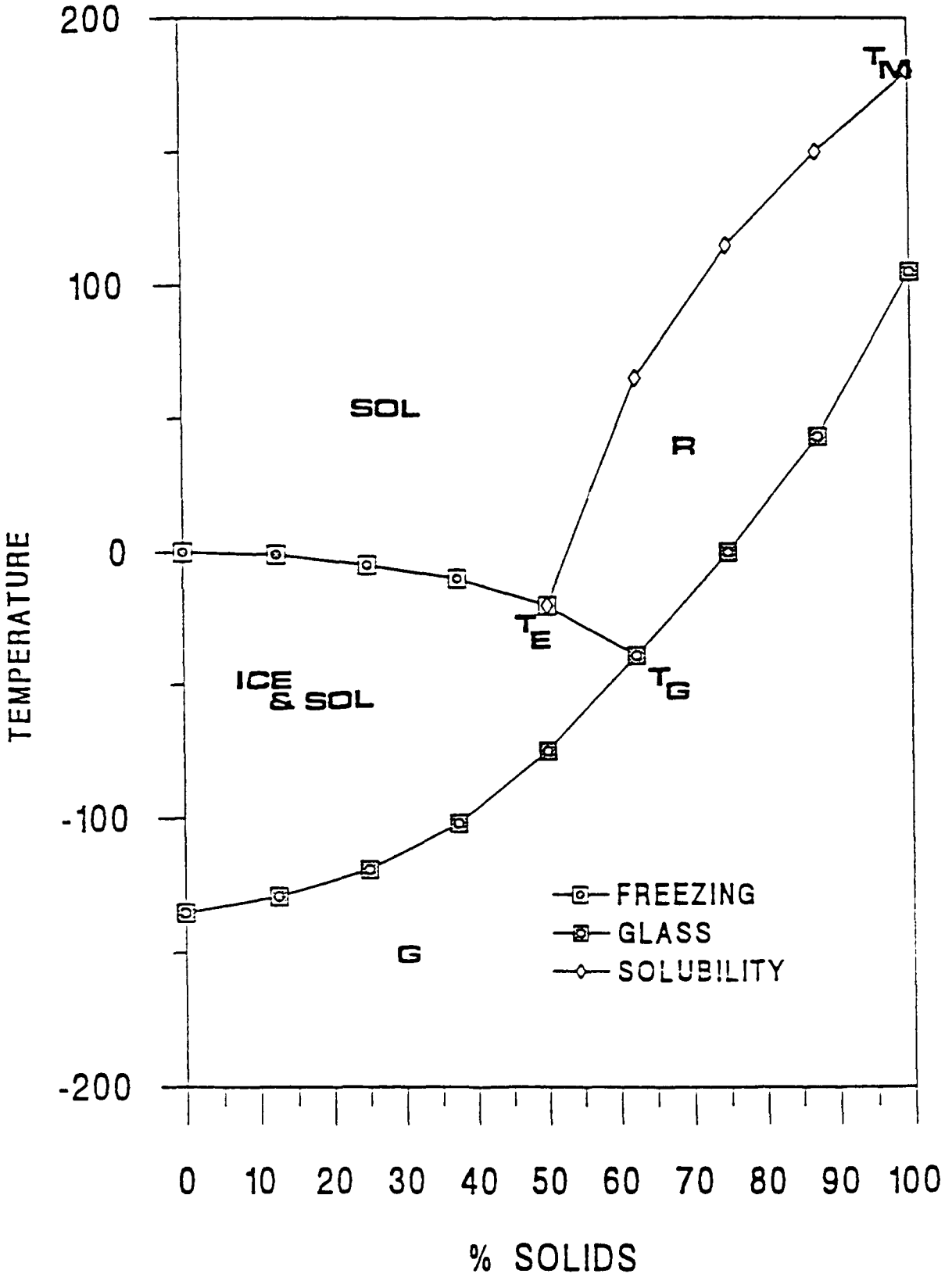

FIG 11. Effect Of Different Storage Temperatures on The Stability Of Red Blood Cells